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(54) Abstract Title
Organic nitrates and nitrites useful against heart disease

(57) The invention relates to compounds for use in the treatment of heart disease comprising a superoxide scavenger and an organic nitrate or nitrite moiety. These compounds do not suffer from the problem of patient tolerance that is associated with the use of conventional agents such as organic nitrates.

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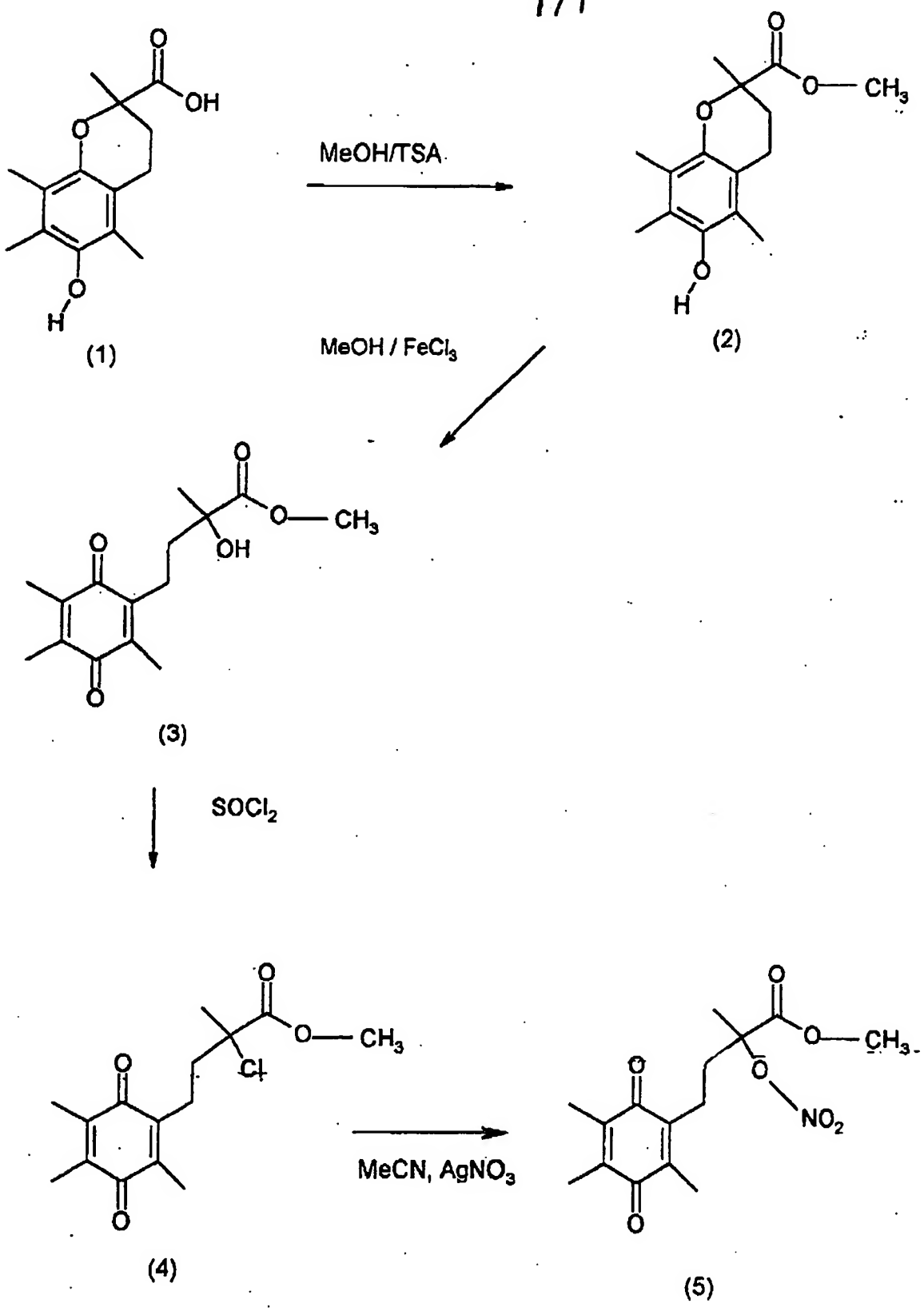
OH
O
O
H
(1)
MeOH/TSA
O
O—CH3
O
H
(2)
MeOH / FeCl3
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O—CH3
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O
O
(3)
SOCl2
O
O—CH3
Cl
O
O
(4)
MeCN, AgNO3
O
O—CH3
O
NO2
O
(5)

FIGURE 1

CHEMICAL COMPOUNDS

The present invention relates to compounds suitable for use in the treatment of heart disease. These compounds do not suffer from the problem of patient tolerance that is associated with the use of conventional agents such as organic nitrates.

Organic nitrates and nitrites have been widely prescribed for the prophylactic treatment of angina for over 100 years. More recently, these drugs have been extended to manage coronary artery disease, acute myocardial infarction and congestive heart failure (Parker & Parker, (1998) *N. Engl. J. Med.* **338**; 520-531). Examples of such drugs include glyceryl trinitrate (GTN), 1,2-glyceryl dinitrate, 1,3-glyceryl dinitrate, isosorbide dinitrate, isosorbide-2-mononitrate and isosorbide-5-mononitrate.

The primary action of organic nitrates is vasodilation, which is attributable primarily to nitrate-induced relaxation of vascular smooth muscle in veins, arteries, and arterioles. Organic nitrates are converted in the body to endothelium-derived relaxing factors (EDRFs), which act to dilate vascular smooth muscle and to inhibit platelet aggregation by activating guanylyl cyclase and increasing intracellular cyclic-3',5'-guanosine monophosphate (cGMP). This represents the cellular basis for the vasodilatory action of organic nitrates.

Organic nitrate administration has been used as a means of providing an exogenous source of EDRF that may help replenish or restore endogenous EDRF levels that are usually impaired in patients with coronary artery diseases such as atherosclerosis.

Discovered to be an EDRF, nitric oxide (NO) is an important endogenous modulator of vascular tone (Ignarro *et al.*, (1987) *Proc. Natl. Acad. Sci USA* **84**:9265-9269; Palmer *et al*, (1987) *Nature* **327**:524-526). A great deal of interest has been shown in the *in vivo* metabolism of organic nitrates to produce NO. However, the cellular mode of action of organic nitrates, in particular, the details of nitrate to NO bio-transformation, still remain unclear. It has been suggested that bio-transformation of organic nitrates to NO is a thiol-dependent enzymatic denitration process catalysed by glutathione-s-transferase and the cytochrome P450-NADPH cytochrome P450 reductase system (Bennette *et al.*, (1994) *Trends Pharmaciol. Sci.* **15**; 245-249). However, it has since been discovered that

glutathione-s-transferase catalyses the reduction of organic nitrate to nitrite, and does not catalyse the reduction of nitrite to NO.

The major problem with nitrate therapy is the rapid development of tolerance and cross-tolerance during repeated dosing with these agents (Parker & Parker, 1998). Clinically, intermittent dosing regimens that allow for a drug-free interval represent the only practical and effective strategy for avoiding nitrate tolerance. Clearly, the need to interrupt drug administration regularly reduces the effectiveness of this form of therapy.

Nitrate tolerance is believed to be a complex multi-factorial phenomenon, and the underlying mechanism of organic nitrate tolerance is poorly understood. One possible route of nitrate tolerance is due to a relative depletion of sulfhydryl groups required for bio-conversion of organic nitrates to NO. More recently it has been suggested that enhanced vascular superoxide production from endothelium plays an important role in this phenomenon (Munzel *et al.*, (1995) *J. Clin. Invest.* **95**:187-194; Rajagopalan *et al*, (1996) *J. Clin. Invest.* **97**:1916-1923).

There thus remains a great need for compounds that are effective in the body as vasodilators and which may be administered continuously for a sustained period of time without suffering a reduction in efficacy due to development of patient tolerance.

According to the present invention there is provided a compound comprising a scavenger of superoxide and an organic nitrate or nitrite moiety. Such compounds are effective vasodilators, yet do not exhibit the problems of patient tolerance to nitrates, from which conventional vasodilatory agents suffer.

The advance that led to the development of the compounds of the invention is based on the inventors' observation of a novel molecular mechanism of the bio-conversion of organic nitrate to NO by xanthine oxidase (XO). The XO enzyme is a homodimer of 150 kDa subunits, and contains four oxidation and reduction centres, one molybdenum cofactor, one flavin adenine dinucleotide (FAD) and two [Fe_2S_2] clusters. XO catalyses the oxidative hydroxylation of a range of aromatic heterocyclic compounds of which the most notable are hypoxanthine and xanthine. During the process of purine metabolism, XO catalyses the two-step oxidation of hypoxanthine, through xanthine, to uric acid. The oxidation of

hypoxanthine or xanthine is concomitantly accompanied by the reduction of oxygen to form superoxide and H_2O_2 (see Table 1, reaction scheme I).

Table 1: Reactions catalysed by XO

Xanthine oxidase activity	$XH + H_2O + O_2 \rightarrow X{=}O + H_2O_2 + O_2^{\cdot-}$	I
NADH oxidase activity	$2NADH + 2O_2 \rightarrow NAD + O_2^{\cdot-} + H_2O_2$	II
Nitrate reductase activity	$2NO_3^{-} \rightarrow NO_2^{\cdot} + O_2$	III
Nitrate reductase activity	$2NADH + 2NO_2^{\cdot} \rightarrow 2NAD + H_2O_2 + 2NO^{\cdot}$	IV

Despite being a reducing agent that is itself capable of causing significant damage to biomolecules, such as by initiating lipid peroxidation, superoxide is considered to be the most important source of oxidative stress. It can be rapidly converted to the highly toxic hydroxyl radical via the Fenton reaction or the Haber-Weiss reaction. It can also react rapidly with NO to form deleterious diffusion-controlled peroxynitrate (Beckman *et al.*, (1990) *Proc. Natl. Acad. Sci. USA* **87**; 1620-1624). Both hydroxyl radicals and peroxynitrate have been shown to initiate lipid peroxidation, protein and enzyme inactivation and DNA fragmentation. On this basis, the classical pathway of superoxide production by XO (see Table 1, reaction scheme I) has been implicated as constituting a major role in a number of pathogenic conditions, such as atherosclerosis, hypercholesterolaemia, diabetes mellitus and rheumatoid arthritis.

In particular, a role of XO in the generation of excess superoxide during hypoxic reperfusion injury has received a great deal of attention (McCord J.M. (1985) *N. Engl. J. Med.* **312**:159-163). During ischaemia, endogenous xanthine dehydrogenase is converted to XO. Concomitantly, hypoxanthine and xanthine are accumulated as a consequence of ATP breakdown. The reperfusion phase following ischaemia allows the XO to use accumulated hypoxanthine or xanthine together with oxygen to produce a burst of tissue-damaging superoxide and H_2O_2.

In addition to the above-described classical reaction of XO, early studies have shown that XO can use NADH as a reducing substrate, possibly binding at a site different to that at which xanthine binds. However, this NADH oxidase activity of XO is not generally recognised and has been little studied over the years.

Several recent studies have suggested that endothelium and vascular smooth muscle contain membrane-bound NADH oxidase enzymes that use NADH as a substrate to produce superoxide (Sanders *et al.*, (1997) *Eur. J. Biochem.* **289**:523-527). The inventors' previous research and that of others has demonstrated that human XO can use not only hypoxanthine or xanthine (Table 1, reaction scheme I) but also NADH (Table 1, reaction scheme II) as a substrate to generate superoxide (Zhang *et al.*, (1998) *Free Rad. Res.* **28**; 151-164).

This NADH-oxidising activity of XO is blocked by diphenyleneiodonium (DPI) but is not suppressible by the conventional xanthine-based inhibitors, such as allopurinol, oxypurinol, BOF-4272 and Amflutizole. Therefore, apart from the xanthine-based free radical-generating pathway, the NADH oxidase activity of XO may operate as an additional pathway to produce free radicals. In other words, XO can contribute to tissue damage depending on which substrate is available in pathological situations.

The third and less well-known phenomenon exhibited by XO is that this enzyme reduces nitrate to nitrite under conditions of low oxygen tension (Fridovich and Handler (1962) *J. Biol. Chem.*, **237**:916-921) although this work was not continued. This property of the enzyme is shared with the assimilatory nitrate reductases of bacteria, but has not attracted significant attention in the literature. Like XO, nitrate reductase also contains both molybdenum and FAD redox centres and utilises NAD(P)H as a reducing substrate.

In a recent paper, the inventors investigated the possible mechanism of nitrate reductase activity of XO by directly detecting NO formation (Zhang *et al.*, (1998) *Biochem. Biophys. Res. Commum* **249**; 767-772). It was found that XO catalyses the reduction of nitrite to NO with NADH as a source of electrons (Table 1, reaction scheme IV). This reductive reaction occurs regardless of environmental oxygen tension, i.e. XO can reduce nitrite in both the presence or absence of oxygen once an electron donor is available. By using two different site-directed XO inhibitors, allopurinol and DPI, it was found that the reduction of nitrite takes place at the molybdenum centre of XO, whilst NADH is oxidised at the FAD centre. This reaction pathway may play a very important role in redistribution of blood flow to

ischaemic tissue by virtue of the vasodilatory effect of NO, since conventional NO synthesis by nitric oxide synthase (NOS) is impaired under ischaemic conditions.

More importantly, the nitrate and nitrite reductase activities of XO has allowed the inventors to speculate a novel explanation for the bio-transformation of organic nitrates to NO in organic nitrate and nitrite therapy of angina pectoris. Indeed, it has been found that XO catalyses not only the reduction of nitrite to NO, but also the reduction of organic nitrates such as glyceryl trinitrate to NO.

During the catalytic reduction of nitrite at the molybdenum centre of XO, oxidation is concomitantly required at the FAD site using NADH as its electron donor. In the absence of oxygen, this reaction will only generate NO from the molybdenum centre. However, in the presence of oxygen, XO exhibits not only nitrite reductase activity (Table 1, reaction scheme IV) but also NADH oxidase activity (Table 1, reaction scheme II). Thus, oxygen will act as an electron acceptor along with nitrite and produce superoxide. Although NO and superoxide may be generated at different redox sites, it is proposed that the simultaneous production of both radicals by the same XO enzyme will result in the formation of peroxynitrate and decrease the net production of NO formation by XO. Increased vascular superoxide production during continuous dosing inactivates NO by forming peroxynitrate, which consequently inhibits NO-mediated vasorelaxation produced by organic nitrates.

Peroxynitrate itself is a non-selective and extremely reactive ion. Its deleterious function inactivates or modifies not only large molecules such as metalloenzymes, but also small molecules, for example, thiols. The formation of peroxynitrate is thus proposed to lead to both the inactivation of XO and the depletion of thiols at one specific enzyme site. The inventors propose that this inactivation, together with mopping-up effect of superoxide on NO explains the hitherto unsolved phenomenon of nitrate tolerance.

As a result of these observations on XO, novel compounds have been designed that are generators of organic nitrates. These compounds are hybrid molecules, comprising both a superoxide scavenger and a generator of NO. The new agents may be expected to diminish nitrate tolerance by at least two mechanisms: (1) the reduction of NO and superoxide interaction and (2) the reduction in superoxide-mediated thiol depletion, by scavenging superoxide produced by NADH oxidation.

The organic nitrate or nitrite moiety should be converted metabolically to NO by endogenous enzymes in the animal body. By animal is meant any animal, although mammals, preferably humans, are considered to be the most appropriate patients for therapy of heart disease. In accordance with the conclusions discussed above, a principle mechanism by which the organic nitrate or nitrite moiety is proposed to be converted to NO is by action of XO in the body. However, any mechanism of metabolic conversion of organic nitrate or nitrite to NO is compatible with the method of action of the compounds of the invention. However, any mechanism of metabolic conversion of organic nitrate or nitrite to NO is compatible with the conversion of the compounds of the invention to NO. In addition, the proximity of the hybrid anti-oxidant scavengers can avert reactive oxygen species-mediated NO consumption or further production of deleterious species.

The superoxide scavenger portion of the compounds of the invention may be any one of a large number of compounds that are known to be effective scavengers of superoxide. Examples include spin-traps such as DMPO, low molecular mass superoxide dismutase (SOD) mimetics such as the $Cu(salicylate)_2$ complex, redox active transition metal complexes such as FeIII EDTA, Zinc carnosine complexes, antioxidants such as desferrioxamine and substrates for direct redox reactions with superoxide such as cytochrome-c and vitamin E. Preferably, the superoxide scavenger is a spin trap that is capable of trapping superoxide. One or more thiol moieties may form part of the superoxide scavenger.

Linkage of the superoxide scavenger to the organic nitrate or nitrite moiety may be through any one of a number of known chemical bonds including ester, amide and ether bonds. Preferably, the linkage itself is resistant to enzymatic degradation. Linkage via a thiol ester is considered to be particularly preferable, since the use of this linkage will reduce the thiol load that is necessary for activation of the organic nitrate moiety in the body. The use of this linkage will thus further minimise tolerance of a patient to organic nitrate-derived NO.

According to a further aspect of the invention, there is provided compound according to claim 1, which is of formula I:

I $(A)_n(B)_m$

where A is a scavenger of superoxide and B is an organic nitrate or organic nitrite moiety. "m" and "n" may be any number, although it is envisaged that the values of m and/or n will not generally exceed about 8. Preferably, m and n are integers.

Preferably, A and B are stably linked. By "stably linked" is meant that the chemical bond that joins the superoxide scavenger moiety and the organic nitrate or nitrite is stable to degradation under physiological conditions. This increases the therapeutic capacity of the compound, since it will not be inappropriately broken down by the metabolism of the body to form compounds that are not functional as either superoxide scavengers or generators of NO. A preferable linkage is a thiol linkage. Ideally, the linkage should also be stable at room temperature and pressure, to increase the shelf-life of the compound. Ideally, the linkage should also be stable or stabilisable at storage temperature.

In order that both the superoxide scavenger and NO-generating functions of the compound are fully exploited, the superoxide scavenger moiety should remain active in trapping superoxide after conversion of the organic nitrate or nitrite to NO.

A particularly preferred compound according to the invention is the nitrate ester (5), the structure of which is illustrated in Figure 1. This compound is proposed to be particularly effective in minimising superoxide interaction with NO upon the conversion of the organic nitrate moiety in the compound.

According to a further aspect of the invention, there is provided a composition containing one or more compounds as described above, in conjunction with a pharmaceutically-acceptable excipient. Suitable excipients will be well known to those of skill in the art and may, for example, comprise a phosphate-buffered saline, a liquid such as water, saline, glycerol or ethanol, optionally also containing mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulphates and the like; and the salts of organic acids such as acetates, propionates, malonates and benzoates. Auxiliary substances such as wetting or emulsifying agents and pH buffering substances may also be present. A thorough discussion of pharmaceutically acceptable excipients is available in Remington's Pharmaceutical Sciences (Mack Pub. Co., N.J. 1991).

According to a still further aspect of the invention there is provided a compound or a composition as described above, for use as a pharmaceutical. Such compounds or

compositions are suitable for the preventative or curative therapy of a wide variety of pathological conditions associated with endothelial dysfunction, in particular of coronary artery diseases such as atherosclerosis and hypertension, and rheumatoid arthritis, diabetes and neurodegenerative diseases. The invention also embraces methods of therapy of heart disease comprising administering to a patient an effective amount of a compound or composition as described above.

Various aspects and embodiments of the present invention will now be described in more detail by way of example, with particular reference to an antioxidant nitrate ester. It will be appreciated that modification of detail may be made without departing from the scope of the invention. All documents mentioned in the text are incorporated herein by reference.

Figure 1 is a reaction scheme illustrating preparation of superoxide scavenger-organic nitrate ester (5).

EXAMPLES

Example 1: Synthesis of antioxidant nitrate ester.

Step 1:

A solution of 2 g (8 mmol) of optically pure (R)-(+)-3,4-dihydro-6-hydroxy-2,5,7,8-tetramethyl-2H-1-benzopyran-2-carboxylic acid (1) and 0.1g of p-toluenesulphonic acid monohydrate in 40 ml methanol are stirred and re-fluxed for 4 hours. After cooling, the solution is diluted with water, extracted three times into ether that is subsequently washed with brine and aqueous sodium bicarbonate solution. The ether solution is washed, dried with $MgSO_4$ and evaporated to give (R)-(+)-methyl 3,4-dihydro-6-hydroxy-2,5,7,8-tetramethyl-2H-1-benzopyran-2-carboxylate (2).

Step 2:

To a stirred solution of 1.5 g (5.68mmol) of (2) in 22 ml ether, is added a solution of 4.5 g (16.6mmol) of ferric chloridehexahydrate in 17 ml of water and 17 ml of methanol. The addition is carried out drop-wise over 30 minutes. After 1 hour the ether layer is separated, and the aqueous phase is further extracted with ether. The combined ether layers are chromatographed on silica gel by flash chromatography using toluene:ethyl acetate as

eluant to afford 1.39 g of the oxidised quinone (R)-(+)-methyl 2-hydroxy-2-methyl-4-(3,4,5-trimethyl-3,6-dioxo-1,4-cyclohexadien-1-yl)butanoate(3).

Step 3:

A solution of 0.5 g of the tertiary alcohol (3) in dry dichloromethane (20 ml) is charged with $SOCl_2$ (5 ml) and stirred at room temperature for 30 minutes. The resulting solution is evaporated in vacuum and re-dissolved in ethyl acetate. This procedure was repeated twice to remove residual $SOCl_2$. The chlorinated product (R)-(+)-methyl 2-chloro-2-methyl-4-(3,4,5-trimethyl-3,6-dioxo-1,4-cyclohexadien-1-yl)butanoate (4) was used without further purification.

Step 4:

To a stirring solution 0.1 g of (4) in dry acetonitrile at room temperature is added one equivalent of silver nitrate ($AgNO_3$). A precipitate of silver chloride forms. The reaction mixture is stirred for a further 30 minutes and is then filtered and evaporated. The nitrate ester (5) is purified on silica gel chromatography using $CHCl_2$ and ethyl acetate as eluant.

Example 2: Exemplification of protective effect of antioxidant.

Buffered solutions containing an organic nitrate as follows [all containing vitamin C between 1 and 5 equivalents: (i) propyl nitrate, (ii) propyl nitrate plus one equivalent of compound (3) and (iii) compound (5) in the range of 1 to 10 μmol are prepared. These solutions are incubated in sealed glass vessels with appropriate concentrations of XO and NADH under both hypoxic and normoxic conditions. After 24 hours levels of NO are measured by aspirating the glass vessels and monitoring NO concentration directly. The results demonstrate the ability of superoxide scavengers to minimise superoxide interaction with NO upon activation of organic nitrates.

CLAIMS

1. A compound comprising a scavenger of superoxide and an organic nitrate or nitrite moiety.

2. A compound according to claim 1, which is of formula I:

$$(A)_n(B)_m \qquad I$$

where A is a scavenger of superoxide and B is an organic nitrate or organic nitrite moiety and n and m are values of between 1 and about 8.

3. A compound according to claim 2 wherein in formula I, n and m are integers.

4. A compound according to claim 3, wherein the values of n and m are both 1.

5. A compound as claimed in any one of claims 2-4, wherein A and B are stably linked

6. A compound according to any one of the preceding claims, wherein said organic nitrate or nitrite moiety forms nitric oxide in the body of an animal.

7. A compound according to claim 6, wherein the nitric oxide is formed by enzymatic conversion of said organic nitrate or nitrite moiety by endogenous enzymes in the body of an animal.

8. A compound according to claim 7, wherein said enzymatic conversion is by xanthine oxidase.

9. A compound according to any one of claims 6-8, wherein the superoxide scavenger remains effective in trapping superoxide upon enzymatic conversion of the organic nitrate or nitrite moiety to form nitric oxide.

10. A compound according to any one of the preceding claims, wherein the superoxide scavenger is a spin trap capable of trapping superoxide.

11. A compound according to any one of the preceding claims, wherein the superoxide scavenger contains one or more thiol groups.

12. A compound according to any one of the preceding claims, wherein the superoxide scavenger is a low molecular mass superoxide dismutase analogue.

13. A compound according to any one of the preceding claims wherein said superoxide scavenger is linked to the organic nitrate or nitrite moiety by a linkage that is stable under physiological conditions.

14. A compound according to claim 13, wherein said linkage is a thiol linkage.

15. Compound (5) in Figure 1.

16. A composition comprising a compound according to any one of the preceding claims in conjunction with a pharmaceutically-acceptable excipient.

17. A compound according to any one of claims 1-15 or a composition according to claim 16, for use as a pharmaceutical.

18. Use of a compound according to any one of claims 1-15 or a composition according to claim 16, in the manufacture of a medicament for the treatment of heart disease.

19. A method of therapy of heart disease comprising administering to a patient a compound according to any one of claims 1-15 or a composition according to claim 16 in an effective amount.

Application No: GB 9910104.0
Claims searched: 1-19

Examiner: Peter Davey
Date of search: 24 July 2000

Patents Act 1977
Search Report under Section 17

Databases searched:

UK Patent Office collections, including GB, EP, WO & US patent specifications, in:

UK Cl (Ed.R): C2C (CMJ)

Int Cl (Ed.7):

Other: Online: CAS ONLINE, WPI, EPODOC, JAPIO

Documents considered to be relevant:

Category	Identity of document and relevant passage	Relevant to claims
E,X	WO 99/37616 A1 (ANGGARD ET AL), 29 July 1999, see eg. claims 1, 3, 8 and 10-12	1 and 16-19 at least

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.